EEDP-04-25
July 1995

Environmental Effects of Dredging Technical Notes

Methods for the Assessment of the Genotoxic Effects of Environmental Contaminants; Cellular and Organ/Organism Effects

Purpose

This technical note is the second in a series of three that outline and describe the principal methods that have been developed to test the potential of environmental contaminants for causing mutagenic, carcinogenic, and teratogenic effects. The first in this series (EEDP-04-24) describes methods used to discern genotoxic effects at the subcellular level, while this technical note describes methods used to discern genotoxic effects at the cellular and organ/organism level. Recent literature citations for each topic are listed in the third technical note (EEDP-04-26) to assist readers in locating source information. A glossary of terms is also provided in Technical Note EEDP-04-26.

The information in these technical notes is intended to provide Corps of Engineers personnel with a working knowledge of the terminology and conceptual basis of genotoxicity testing. To develop an improved understanding of the concepts of genotoxicity, readers are encouraged to review "A Primer in Genotoxicity" (Jarvis, Reilly, and Lutz 1993), presented in Volume D-93-3 of the *Environmental Effects of Dredging* information exchange bulletin.

Additional Information

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Cytogenetics (Refs. 1-10)*

Cytogenetics is the study of genetic damage that is discernible at the cellular level. Cytogenetic procedures often involve isolating cells from exposed animals or using tissue slices from specific organs and analyzing them for gross abnormalities. Commonly used cytogenetic assays are the micronucleus assay, sister chromatid exchanges, and chromosome aberrations.

To realize how toxicants can affect cells, it is essential to have a basic understanding of cell division. Mitosis is the cell division of somatic cells, that is, all cells except the sex, or germ, cells. The mitotic cycle has five stages: interphase, prophase, metaphase, anaphase, and telophase. The only visible nuclear changes in interphase are the increasing volumes of the nucleus and nucleolus. In prophase, nuclear division and contraction by coiling of the chromosomes has started. Individual chromosomes can be distinguished and are seen to be double. By the end of this phase, the nucleoli shrink and disappear. In prometaphase, the spindle is organized and some fibers run from each pole to the centromeres of each chromosome. In the short time of metaphase, the centromeres are usually aligned along the equator, and daughter centromeres are still attached to each other. The coiling of chromatids is completed. In the anaphase stage, the division of centromeres is completed and the centromeres of each chromosome pair separate. Movement ceases when all the centromeres are aggregated closely about the poles. In telophase, the nuclear envelope reforms as do the nucleoli. Telophase is completed when the nuclear membrane is finished and the nucleoli have reached full size, creating two complete cells.

Micronucleus Assay (Refs. 119-132)

Micronuclei are small, secondary nuclei formed during telophase in cell division. They develop from chromatin (DNA and associated nucleoproteins) lagged in anaphase resulting from chromosome breakage or a malfunction of the spindle apparatus. To assay for micronuclei, cells either from tissues of exposed animals or grown in culture and exposed in vitro are isolated and fixed on microscope slides. The cells are stained with Giemsa, which allows visualization of nuclei using microscopy. The percentage of micronuclei cells in the exposed animal is compared to that of a control animal. An increase in the frequency of micronucleated cells present in a tissue is an index of chromosome damage associated with exposure to a genotoxic agent.

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* Refer to bibliographic citations 1 through 10 in *Environmental Effects of Dredging* Technical Note EEDP-04-26.

Sister Chromatid Exchanges (Refs. 133-143)

During cell division, the chromosomes in the parent cell divide into two chromatids and replicate themselves. Each of the two daughter cells formed receives one of the parent chromatids in addition to the newly created, replicate (sister) chromatid. A sister chromatid exchange (SCE) is a mutagenic event in which an exchange of chromatin occurs between two sister chromatids at the same locus, caused by a break in both of the DNA strands. An in vitro SCE assay is typically performed using Chinese hamster ovary (CHO) cells since they contain a small number (21) of relatively large chromosomes.

In the SCE assay, CHO cells are incubated with the test chemical for 2 hr, and the exposure is terminated by changing the cell growth medium. The treated cells are then incubated for 24 to 30 hr with 5-bromo-2'-deoxyuridine, a fluorescent analog of deoxyuridine, a precursor of one of the four DNA bases, deoxythymidine. During this incubation time, the fluorescent base is incorporated into the daughter chromatid. The cells are fixed onto microscope slides and stained with Giemsa and Hoechst 33258, which allows visualization of the chromosomes upon exposure to ultraviolet light. Figure 1A illustrates a typical fluorescence pattern of control cells, with the daughter chromatid containing almost all of the incorporated fluorescent base (dark chromatids). Note that background SCEs do occur. Figure 1B illustrates a typical fluorescence pattern of exposed cells.

Chromosome Aberrations (Refs. 144-155)

Chromosome aberrations are the formation of chromosomes that are different from the original chromosomes. The expression of chromosome damage is usually dependent on cells performing DNA replication and nuclear division. These changes can occur structurally and numerically. Structurally, a normal chromosome consists of a centromere that determines the morphology of the

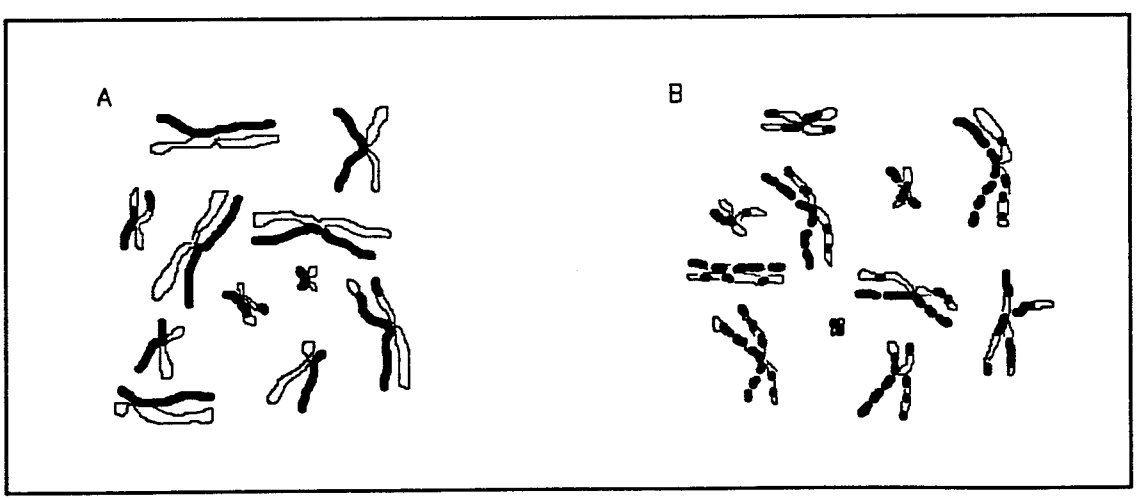

Figure 1. Sister chromatid exchanges in control cells (A) and cells treated with a mutagen (B)

chromosome and two chromatids, each composed of two complementary DNA strands. There are three distinct normal shapes of chromosomes: metacentric (V-shaped), submetacentric (J-shaped), or acrocentric (I-shaped).

Structural aberrations of chromosomes include chromosome breakage, inversion, and translocations. Chromosomal breakage includes single chromosome breaks with fragments, double breaks with deletion, duplication, or ring chromosome formation as shown in Figure 2. Inversion occurs when breaks in the chromosome swing around 180 deg and rejoin at the ends, and the whole segment of the chromosome lies in inverse genetic order.

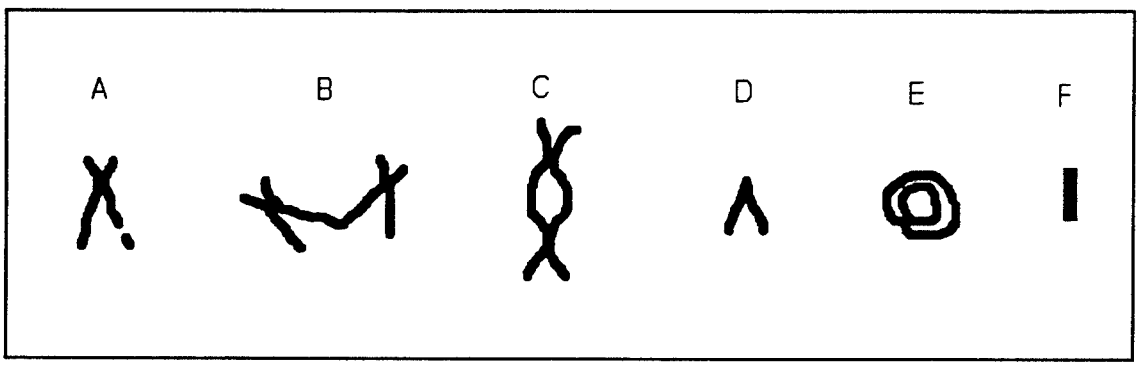

Figure 2. Types of chromosome aberrations: (A) chromatid deletion, (B) triradial chromatid exchange, (C) dicentric chromosome, (D) acentric fragment, (E) centric ring, and (F) small interstitial deletion

Sometimes in cell division, chromosomes have an abnormal number of centromeres. One sister chromosome will have two centromeres and is called dicentric while the other chromosome does not have a centromere and is termed acentric (also illustrated in Figure 2). Dicentric and acentric chromosomes are considered unstable because they are frequently lost from cells when two centromeres proceed to the opposite poles in cell division.

Another type of chromosomal aberration is change in the chromosome number. Aneuploidy is the gain or loss of chromosomes in which the chromosome number differs from the normal haploid (n) or diploid (2n) chromosome number. Aneuploidy usually occurs as a consequence of errors in nuclear division. Nondisjunction contributes to aneuploidy and is a chromosome loss that occurs because of anaphase lagging. Also with anaphase lagging, one or more chromosomes can be lost because some of the chromosomes may fail to move to the poles of the cell during anaphase and will thus be left out of the nucleus when the nuclear membrane is reformed. Nonspecified metaphases occur when the morphology of the chromosomes is not well defined.

Polyploidy occurs when a diploid gamete unites with a normal haploid gamete during fertilization and a triploid (3n) zygote results.

Histopathology (Refs. 156-179)

Histopathology offers biomarkers that integrate the adverse biochemical and cellular effects of xenobiotics to indicate whether target organs/organisms have been compromised. Biochemical alterations can cumulate into cellular alterations which, in turn, can cumulate into histopathological alterations (lesions) of organ systems. Thus, histopathology provides a means of monitoring the actual health of aquatic species, rather than using biochemical and cellular alterations to predict possible adverse effects.

Drawbacks of histopathology include the inability to ascertain specific etiologic agents and the uncertainty of distinguishing lesions caused by infectious disease, normal physiologic variation, or natural toxins from lesions caused by anthropogenic chemicals. Also, the organism must be sacrificed for histological examination, eliminating the possibility of time-sequenced examination of the same organism.

Histopathology involves sacrificing the subject organism and isolating the organ to be examined. The tissue is preserved with a fixative (formalin, for example) and prepared for sectioning. Preparation for tissue sectioning usually entails dehydrating the tissue by placing it in a series of alcohols and solvents and infiltrating the tissue with paraffin to fill in the dehydrated spaces of the tissue. The tissue is then embedded in paraffin and sectioned using a microtome, which is capable of slicing sections 1 µm thick. The thin tissue sections are mounted onto microscope slides and stained for microscopic examination. The most common types of damage that are looked for are described below.

While other organs experience lesions and are examined histopathologically, the liver is the most-used organ for such studies. The liver is highly perfused with blood, receiving the highest percentage of cardiac output of all organ systems (~ 27 percent, depending on species). Chemicals absorbed from the diet (nutrients and xenobiotics) are transported directly to the liver for "processing" before they are distributed to other parts of the body. The liver is rich in xenobiotic metabolism enzymes, and most phase I and II metabolism occurs there. If reactive metabolites are formed, most react in the liver, the site of metabolism. Therefore, the liver is the most probable initial site of action for most toxicants.

Hepatocellular Necrosis

Hepatocellular necrosis is, by definition, death of liver cells, or hepatocytes, and usually occurs as the result of a sudden cessation of blood flow or damage by toxic agents. Necrotic changes often are focal or multifocal, in that they occur in localized areas within the organ. The liver consists of approximately 40 different cell types, which vary widely in their purpose. Foci of necrotic cells may be observed in, for example, cells with high cytochrome P450 content, indicating possible exposure to xenobiotics. Toxicant-related hepatic

necrosis must be differentiated from necrosis due to postmortem changes. As such, stringent sampling and tissue fixation protocols are required.

Hyperplasia of Regeneration

After hepatocellular necrosis occurs, if the organism survives, the remaining hepatocytes undergo hyperplasia of regeneration, replacing necrotic cells. The replacement cells are smaller, irregularly shaped cells which form islands at the foci of the necrotic cells. These areas of hyperplasia of regeneration are used as indicators of prior hepatocellular necrosis.

Hepatocytomegaly

Hepatocytomegaly is an enlargement of the hepatocytes and is generally classified into three types: hepatocellular hypertrophy, megalocytosis, and hepatocellular vacuolation. Hepatocellular hypertrophy is an enlargement of cellular diameter without accompanying nuclear changes, leading to a net gain in the dry mass of the liver. A common cause of hepatocellular hypertrophy is proliferation of endoplasmic reticulum, indicating induction of cytochrome P450, that is, exposure to cytochrome P450-inducing compounds. Megalocytosis is characterized by enlargement of both the cell and the nucleus, and hepatocellular vacuolation is characterized by vacuolation, or formation of pockets of fluid within the hepatocytes. Little is known about the mechanism of the latter two types of hepatocytomegaly, but all three types are associated with exposure to genotoxic contaminants.

Foci of Cellular Alteration

Foci of cellular alteration, also known as staining or tinctorial changes, become apparent upon staining of liver sections. The conventional stains used in histopathology, hematoxylin and eosin, stain hepatocytes different colors and different intensities of color depending on cellular content. Hematoxylin is a basic dye, and cells that it stains are termed basophilic. Basophilic cells are depleted of glycogen and have increased levels of cytoplasmic RNA, both indicating protein synthesis. Eosin is an acidic stain that stains eosinophilic cells, which are generally hypertrophic and have a reduced glycogen content. Clear cells are cells that stain with neither hematoxylin nor eosin and are glycogen rich. Foci, or localized areas of similarly staining cells, can indicate areas of toxicant effect.

Foci of Enzyme Alteration

Foci of enzyme alteration is a newer technique based upon foci of cellular alteration. This technique uses a different tissue sectioning method: cryostatically sectioned tissue slices. Rather than paraffin-embedding the liver, it is placed in a mold and embedded in a glycol/resin compound that hardens to about the same consistency as paraffin upon freezing. The frozen tissue is then sectioned using a cryostat, which is basically a microtome in a freezer.

The frozen tissue sections are mounted onto microscope slides and still maintain their enzyme functions.

Determination of foci of enzyme alteration may be accomplished using histochemical, immunohistochemical, and in situ hybridization techniques. Histochemical techniques involve flooding the tissue section with a particular substrate for the enzyme of interest that is an irreversible inhibitor of the enzyme; that is, the substrate covalently binds to the enzyme. A radiolabeled, colored, or fluorescent substrate is used, and the foci of enzyme alteration (for example, induction of a particular enzyme) can be noted with light microscopic autoradiography, light microscopy, and fluorescence microscopy, respectively. Light microscopic autoradiography involves dipping the slides into a special photographic emulsion in the dark and developing the slides with photographic fixer and developer, which stains the bound radiolabeled substrate with silver grains. Immunohistochemical techniques utilize the antigen-antibody principle, in that an antibody to a specific enzyme is constructed and incubated with the tissue section. The antibody binds to the enzyme/antigen and is visualized using the same means as with the histochemical techniques. Both histochemical and immunohistochemical techniques assay for enzymes, with the immunohistochemical techniques being more specific for a particular enzyme.

In situ hybridization examines enzyme alteration at the molecular level rather than at the protein level. If a protein/enzyme is induced in response to toxicant exposure, messenger RNA (mRNA) specific for that protein increases within the cell, which then is translated into the protein. A probe for that particular mRNA, which is a length of complementary RNA (cRNA) that will hybridize (or noncovalently bind) to the mRNA, is used that is radiolabeled or fluorescent labeled. The probe is incubated with the tissue section for hybridization and visualized using the appropriate technique (light microscopic autoradiography or fluorescence). Foci of enzyme alteration, like foci of cellular alteration, can indicate genotoxicant exposure.

Neoplasms

Neoplasms are cancers or tumors and are one of the least-desired contaminant effects in aquatic populations. Therefore, neoplasms are a signal of definite genotoxic contamination, while the biomarkers discussed earlier are signals of potential genotoxic contamination. Several types of hepatic neoplasms are associated with genotoxic contaminant exposure and are named based on the type of tissue in which they occur. Hepatic adenomas, hepatocellular carcinomas, cholangiomas, and mixed hepato-cholangiocellular carcinomas are the most important such neoplasms. Neoplasms are diagnosed either through histopathological methods or by gross autopsy.

Nonhepatic Tissues

Tissues other than the liver are also used histopathologically to assess genotoxicant exposure. Ovary and sperm morphology, skin neoplasms, and spleen,

kidney, and brain histopathologies are also used to some degree, as these are sites of action of certain specific toxicants.

Developmental Abnormalities (Refs. 180-201)

Embryonic development can be considered a weak link in the life cycle of an organism because, during this period, distinctive cellular and molecular processes operate to form a complex multicellular organism from an embryo. These processes can be easily disturbed by many chemicals. Developmental toxicants exert their effects on embryos at concentrations lower than those required to affect adults or cause general cellular toxicity.

One type of assay used to assess developmental effects in aquatic organisms is early life stages (ELS) testing. ELS involves exposing organisms at the time of fertilization until some later time period and observing for hatching success, survival and growth of larvae, and often, deformities of larvae. Organisms commonly used for ELS testing include Japanese medaka, rainbow trout, and the South American clawed frog (*Xenopus laevis*) (the FETAX, frog embryo teratogenesis assay-*Xenopus*).